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(54) Title: H-Y DERIVED EPITOPES AND USES THEREFOR (57) Abstract We show that one human H-Y antigen presented by HLA-B7 is an 11 residue peptide derived from <i>SMCY</i> , an evolutionarily conserved Y chromosomal protein. A homologous gene on the X chromosome, <i>SMCX</i> , differs by two residues in the same region. The identification of H-Y offers prospects for improvements in transplantation outcome, prenatal diagnosis and fertilization strategies.		

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H-Y DERIVED EPITOPES AND USES THEREFORMention of Government Grant

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BACKGROUND OF THE INVENTIONField of the Invention

This invention relates to the field of immunology, especially as it concerns the transplantation of organs, tissues and cells.

Description of the Background Art

In 1989, a major human organ was transplanted into another human being every 40 minutes. The 16,000 organs transplanted that year included almost 9,000 kidneys, nearly 2,000 each of hearts and livers, and close to 3,000 bone marrow transplants.

Such transplants, which are from an animal of one species to another animal of the same species, are termed allografts.

A significant practical limitation on the practice of allograft therapy is the acute shortage of donor organs of all types. This shortage is exacerbated by the need to match donors and recipients for immunocompatibility. Immunological differences between donor and recipient, even though of the same species, can lead to immune rejection of a transplant (graft). In addition, where a graft contains immunocompetent cells, these can recognize "host" antigens as foreign, leading to an adverse immune reaction known as graft-vs.-host disease.

The antigens which differ from one individual to another, and which are most likely to elicit these responses, are called "histocompatibility" or "transplantation" antigens. Histocompatibility antigens that can induce transplant rejection include the class I and class II molecules of the major histocompatibility complex (MHC), as well as a large number of so-called minor histocompatibility antigens (mHag). In mice, the use of inbred strains has shown that mHag are encoded by almost 50 different allelically polymorphic loci scattered throughout the genome (1). In humans, mHag have been shown to exist, although their overall number and complexity remains

uncertain.

The antigens of the "major" histocompatibility complex lead, if mismatched, to rapid graft rejection. The so-called "minor" histocompatibility or transplantation antigens also
5 stimulate graft rejection, although at a slower pace than for the MHC antigens. These reactions can be life-threatening and therefore are not "minor" from a clinical standpoint (1). Even when donors and recipients are perfectly matched as determined by HLA serotyping and by mixed leukocyte culture tests, moderate
10 to severe acute graft-vs.-host disease occurs in 30-70% of bone marrow transplant recipients and is the primary or contributing cause of death in 15-40% (6).

Attempts have been made to control graft rejection and graft-versus-host disease by administering general
15 immunosuppressants and, in the case of bone marrow transplants, in vitro depletion of donor marrow of T cells pretransplant. However, these strategies have the disadvantage that the immune system of the patient is more or less compromised, leaving the patient vulnerable to various diseases.

20 *The Role of the H-Y antigen in Graft Rejection and Graft-vs.-Host Disease.* In both mice and humans, one of the most interesting of the mHag has been the male specific antigen, H-Y. H-Y was identified through the observation that within an inbred mouse strain, most of the male-to-female skin grafts were
25 rejected, whereas transplants in other sex combinations nearly always succeeded (2). A similar antigen exists in humans (3), and further studies have established that sex mismatch is a significant risk factor associated with graft rejection or the development of Graft-versus-host disease in bone marrow
30 transplant recipients (3-6). H-Y mismatch can lead to rejection of HLA-matched male organ and tissue grafts by female recipients, as well as of female bone marrow grafts by male recipients.

The H-Y antigen is ubiquitously expressed in different
35 human tissues (4,7), and H-Y specific immune responses occur during the transplantation of organs, blood transfusion, and pregnancy (reviewed in 8). However, the origin and function of H-Y antigens has eluded researchers for 40 years.

As with other mHag, the recognition of H-Y by T lymphocytes is MHC-restricted (3,9), and it has been shown that some H-Y antigens are peptides derived from cellular proteins that are presented on the cell surface in association with MHC class I molecules (10).

Attempts to Identify the Gene Encoding the H-Y Antigen, or to Characterize the H-Y Antigen. Even though the H-Y antigen was first discovered in 1955, the gene encoding the H-Y antigen has not previously been identified, and this has hampered the development of certain immunological solutions to the health risks posed by H-Y antigen-mediated graft rejections.

The human H-Y gene has been mapped to the long arm of the Y chromosome (21), while the mouse H-Y gene is believed to reside on "deletion interval 2" of the short arm of the murine Y chromosome (23). The latter paper suggested that at least two and up to five distinct loci encode H-Y antigens.

Peptides have been extracted by acid elution from lysates of splenocytes of male and female mice and separated by reverse-phase HPLC. H-Y-specific CTLs (11P9 cell line) recognized fraction 28 from the male cell lysate, but not from the female lysate, suggesting that this fraction contained a CTL epitopic fragment of the H-Y antigen (10). However, this fraction was not further resolved or characterized.

The SMCX and SMCY Genes. A mouse gene, smcy, was found in the course of a search of deletion interval 2 of the short arm of the Y chromosome (19). The authors speculated as to the possibility that this gene encoded various antigens, including the H-Y antigen, the "serologically detected male antigen" (SDMA), and a spermatogenesis factor (Spy). A related gene, smcx, which is found on the X chromosome, was also characterized. Agulnik, et al., Id., 879-84. The genes are 78% identical at the nucleotide level. The translation products are 82% identical over a 321 amino acid stretch.

Identification of T cell epitopes in Proteins. By combining microcapillary liquid chromatography/electrospray ionization mass spectrometry with T cell epitope reconstitution assays, we previously identified peptide antigens recognized by T cells specific for human melanoma (11; and see PCT/US95/01991,

USSN 08/234,784 and USSN 08/197,399), human xenografts (12), and a non-sex-linked human mHag (13). However, the identification of a CTL epitope of an H-Y antigen has not previously been reported.

5 Cytel, WO94/03205 discloses methods for identifying HLA binding peptides.

No admission is made that any reference cited in this specification constitutes prior art, or pertinent prior art. Applicants reserve the right to challenge the cited date of
10 *publication, or the accuracy of the data reported in any reference, if they are given reason to believe that these are incorrect or misleading.*

SUMMARY OF THE INVENTION

Applicants have identified a peptide fragment recognized
15 by a human cytotoxic T lymphocyte (CTL) clone that is H-Y specific and restricted by the class I MHC molecule HLA-B7. It is believed that this fragment represents a CTL epitope of the H-Y antigen. Such a fragment, and various derivatives, mutants, and conjugates of the fragment, are of value in
20 predicting the likelihood of a severe graft-versus-host reaction to a transplant, as well as in preventing or ameliorating such a reaction.

Other embodiments and uses are set forth in this specification.

25 BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Reconstitution of the H-Y epitope with HPLC fractionated peptides extracted from HLA-B7 molecules. (A) HLA-B7 molecules were immunoaffinity purified from 2×10^{10} H-Y positive JY cells. Peptides were eluted from B7 molecules with
30 10% acetic acid, pH 2.2, filtered through a 10 kD cut-off filter and fractionated on a C18 reverse phase column. Buffer A was 0.1% heptafluorobutyric acid (HFBA); buffer B was 0.1% HFBA in acetonitrile. The gradient consisted of 100% buffer A (0-20 min), 0 to 12% buffer B (20 to 25 min), and 12 to 50% buffer B
35 (25 to 80 min) at a flow rate of 200 μ l/min. 60 fractions of 200 μ l each were collected from 20 to 80 min. (B) Fractions 28

and 29 from the separation shown in (A) were rechromatographed with the same acetonitrile gradient, but using trifluoroacetic acid (TFA) instead of HFBA as the organic modifier. For both panels, 3% of each peptide fractions were preincubated with
5 1,000 ^{51}Cr -labeled T2-B7 cells at room temperature for 2 hours. CTLs were then added at an effector to target ratio of 10 to 1, and further incubated at 37°C for 4 hours. Background lysis of T2-B7 by the CTL in the absence of any peptides was -3% in (A) and -4% in (B); positive control lysis of JY was 75% in (A) and
10 74% in (B).

Fig. 2. Determination of candidate H-Y peptide by mass spectrometry combined with ^{51}Cr release assay. HPLC fraction 14 from the separation shown in Fig. 1B was chromatographed with an on-line microcapillary column effluent splitter as previously
15 described (11,13). One-fifth of the effluent was deposited into 100 μl of culture media in microtiter plate wells for analysis with CTLs as in Fig. 1. The remaining four-fifths of the material were directed into the electrospray ionization source, and mass spectra of the peptides deposited in each well were
20 recorded on a triple-quadrupole mass spectrometer (Finnigan-MAT, San Jose, California). (\diamond), H-Y epitope reconstitution activity measured as percent specific lysis; (\blacksquare), abundance of peptide 1171 measured as ion current at m/z 391.

Fig. 3. CAD mass spectrum of peptide 1171 after conversion
25 the R residue to ornithine. Material from second dimension HPLC fraction 14 shown in Fig. 1B was treated with 70% hydrazine hydrate for 1 hour. The CAD mass spectrum was recorded on the $(M+2H)^{+2}$ ion at m/z 566.

Fig. 4. H-Y epitope reconstitution with synthetic
30 peptides. Synthetic peptides were purified to homogeneity by reverse phase-HPLC on a Vydac C4 column. Purity was established on an analytical RP column and the quantity of each peptide was confirmed by comparing the area of the peak with that of a standard peptide. The identity of the peptides was confirmed
35 by mass spectrometry. ^{51}Cr release was assayed at an effector to target ratio of 10 to 1 on T2-B7 cells that had been incubated with the indicated concentration of SMCY peptide SPSVDKARAEEL (\bullet), or SMCX peptide SPAVDKAQAEEL (\blacksquare).

Fig. 5. Binding of synthetic peptides to purified HLA-B7. HPLC-purified test peptides were assayed for the ability to inhibit the binding of the iodinated endogenous B7 peptide APRTYVLLL to purified HLA-B7 as previously described (26). ♦, SMCY peptide SPSVDKARAEI; ■, SMCX peptide SPAVDKAQAEL; ▲, APRTLVLILL, an endogenous peptide bound to HLA-B7; X, LLDVPTAAV, an endogenous peptide bound to HLA-A2.1 as the negative control.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

The present invention facilitates the inhibition of an adverse immune reaction to an H-Y antigen. Since the H-Y antigen is male specific, such a reaction is most often encountered when a female receives a donation of a male-derived organ or tissue transplant. However, if a transplant from a female donor to a male recipient includes immunocytes, as would be the case with a bone marrow transplant, then the transplanted T cells could react adversely to the H-Y+ cells native to the recipient. One of the purposes of the present invention, therefore, is to present an H-Y specific CTL epitope, as hereafter defined, to T cells in such a manner as to tolerize the T cells against a foreign H-Y antigen, thereby muting the immune response which would otherwise occur as a result of the transplantation.

Such tolerization may be achieved by any means known in the art. One such means is to repeatedly administer to the recipient very small doses of the immunogen. If desired, the size of the doses may be increased from time to time as the patient's ability to tolerate the immunogen increases. Another means is to administer the immunogen by a route of administration, such as the oral route, which is less effective, immunogenically, than the intravenous route. See Weiner, et al., Ann. Rev. Immunol., 12: 809-37 (1994); Friedman, et al., Chem. Immunol. (Switz.) 58: 259-90 (1994).

Another possibility is to administer an H-Y specific TCR antagonist. Certain antigen analogues have been found to be more efficient at inhibiting T cell activation by the parental antigen than are unrelated peptides. It has been speculated

that the TCR antagonist: MHC molecule complex competitively blocks the T-cell receptor so that it is nonresponsive to processed H-Y antigens, yet does not interact with the receptor in such a manner as to cause T cell activation.

- 5 A partial agonist could also be useful. These can signal T cells for lymphocyte production but are unable to elicit the signals required for proliferation, presumably because of some abnormality in their interaction with the T-cell receptor.

For a review of TCR antagonists and partial agonists, see
10 Sette, et al., Ann. Rev. Immunol., 12: 413-34 (1994), and see also the references cited therein. Other relevant publications include Eckels, et al., WO90/08161 and Gefter, et al., WO88/00057, and the references cited therein (including references cited in the attached International Search Reports).

- 15 Yet another means of inducing tolerance is to block one or more of the pathways involved in the adverse immune response. For example, one may blockade the B7-CD28 or B7-CTLA4 pathway using anti-CD28 or anti-CTLA4 antibodies, and/or B7 analogues which act as antagonists by blocking the CD28 or CTLA4 binding
20 site. See Boussiotis, et al., Curr. Opin. Immunol. 6: 797-807 (1994).

The H-Y specific epitope may be presented on an incomplete antigen-presenting cell, such as a T cell genetically engineered so as not to express CTLA4, or a fibroblast cell genetically
25 engineered to express the necessary MHC molecule but not other molecules necessary for effective antigen presentation.

If desired, two or more means of tolerization may be used in combination, either simultaneously or sequentially, in order to achieve a greater degree of tolerance, or to achieve
30 tolerance more rapidly, or to achieve it with fewer side effects. When such means are combined, it should be understood that the elements of the combination may be such that, if used alone, they would be ineffective.

An immune reaction to a foreign H-Y antigen may also
35 complicate blood transfusions. The methods of the present invention may, mutatis mutandis, be adapted to the inhibition of serum sickness attributable to a transfusion between

individuals of different sexes.

Since H-Y reactions can occur in pregnancy (when a pregnant woman is bearing a male fetus), the methods of the present invention may also be helpful in damping the immune response of
5 the mother to the fetal H-Y antigen.

The compositions of the present invention also have diagnostic value. For example, they can be used to assess the degree of chimerization of the bone marrow of a patient who has received a sex-mismatched bone marrow transplant; the post-
10 transplant marrow may include both H-Y+ and H-Y- T cells.

Transplantation

The list of organs which may be transplanted is very long and is primarily limited by known surgical techniques. Certain tissues or parts of organs, as well as cells which are not
15 organized into organs or tissues, also may be transplanted. For the purpose of the appended claims, the term "organ" includes whole organs, parts of organs, and miscellaneous tissues. Examples include kidney, eye, heart, heart valve, skin, liver, bone marrow, intestine, blood vessels, joints or parts thereof,
20 pancreas or portions containing the islets, lungs, bronchi, brain tissue, muscle and any other vascularized tissue. However, the transfusion of blood or components thereof is not be construed as being an organ transplantation.

Transplantation may be performed to correct an organ which
25 is missing, incomplete, or improperly functioning as a result of injury, genetic defect, disease, toxic reaction, etc. The recipient may receive the transplanted organ to supplement existing tissue such as using skin tissue to treat burns, pancreatic islets for diabetes or brain tissue for Parkinson's
30 disease. Alternatively, the recipients defective organ may be completely removed and replaced with the graft such as in kidney, heart, liver, lung or joint transplants. For some organ transplants, either approach may be used, such as with liver transplants for treating cirrhosis and hepatic neoplasms
35 and infections.

H-Y-Specific CTL Epitopes

The H-Y-specific CTL epitopes of the present invention are

peptides, typically 9-13 amino acids in length, which are sufficiently similar to an H-Y epitope recognized by an H-Y specific CTL to be useful, under suitable conditions of use, as tolerogens to protect an individual from an H-Y specific
5 allogeneic response, or to be useful in the diagnosis of the susceptibility of an individual to such a response. Preferably, such epitopes are identical to or otherwise substantially homologous with H-Y-specific peptide epitopes recognized by H-Y-specific CTLs. Applicants believe that SMCY is likely to be
10 either an H-Y antigen, or at least substantially homologous with an H-Y antigen. Consequently, these peptides may be identical to, or otherwise substantially homologous with, SMCY (which appears to be one of the human H-Y antigens) and especially to the identified SPSVDKARAEL epitope. Since, in turn, SMCX is
15 substantially homologous with SMCY, they may also be identical or otherwise substantially homologous to SMCX (especially to its SPAVDKAQAEEL sequence).

The family of H-Y epitopes which are recoverable from an individual is dependent on the nature of the binding site of the
20 Class I MHC (HLA) molecules expressed by the individual, and, as a result of the polymorphism of the Class I MHC (HLA) molecules, can vary considerably from one individual to another. For the purpose of the present invention, the H-Y cell line used as a source of H-Y-specific CTL epitopes may be any H-Y cell
25 line; similarly, the Class I MHC (HLA) molecule may be any such molecule which is capable of binding to and presenting a H-Y-specific epitope, including, but not limited to, the various allelic forms of Class I MHC molecules. Among the Class I molecules, the principal genetic loci are denoted as HLA-A, HLA-B, and HLA-C. The H-Y specific epitope of the present
30 invention may be HLA-A, HLA-B or HLA-C restricted.

The preferred epitopic sequence may vary depending on the restriction system. Preferably, the H-Y specific epitope is one restricted by one of the more prevalent (at least among the
35 intended patient population) allelic forms of these loci. Among HLA-A, the principle alleles are HLA-A2, -A3, -A1, -A11 and -A24. Among HLA-B, the most prevalent alleles are HLA-B7 and -B8; however, HLA-B is more polymorphic than HLA-A. Epitopes

presentable by HLA-B7 are of particular interest.

Preferably, the peptide concentration at which the epitope-stimulated CTLs achieve half the maximal increase in lysis relative to background is no more than about 1 nM, more preferably no more than about 100 pM, still more preferably no more than about 10 pM. For the peptide SPSVDKARAEL, the potency was 10 pM. Even when a peptide is being used as a tolerogen, high potency is advantageous as it permits use of low concentrations of the peptide, which, besides being more economical, also reduces the chance of a side effect.

Preferably the epitope is recognized by CTLs from at least two different individuals, more preferably at least five different individuals.

More preferably, the CTL epitope satisfies two or more of the above desiderata.

In a preferred embodiment, the H-Y epitope of the present invention has the sequence Ser-Pro-Ser-Val-Asp-Lys-Ala-Arg-Ala-Glu-Leu (SPSVDKARAEL), or a functional fragment thereof.

Assuming that SMCY is identical to or otherwise substantially homologous with at least one H-Y antigen, other H-Y epitopes may be identified by systematic study of the immunological properties of 9-13 amino acid fragments of SMCY. Since, until the present discovery, the connection of SMCY to H-4 was highly speculative, and SMCY had no known biological function, the art lacked the motivation to make such a study. Alternatively, other H-Y epitopes may be identified as was SPSVDKARAEL, but by screening with other H-Y specific CTLs, especially those restricted by MHCs other than HLA-B7.

This peptide, although recognized by HLA-B7-restricted H-Y-specific CTL, may not be optimal at present. It is known that some residues on CTL epitopes are particularly important for binding of the peptide to the MHC molecule, while others are particularly important for Tc recognition. The other residues may be important for either or both. (refs: E.L. Huczko et al. J. Immunol. 151:2572, 1993; J. Ruppert et al. Cell 754: 929, 1993; Madden Dr et al. Cell 75:693-708, 1994.) It is proposed that amino acid substitutions for the peptide may be useful at altering the immune response to the peptide. Using existing

knowledge about which of these residues may be more likely to affect binding either to the MHC or to the TcR, a rational approach to this process may be employed. The resulting peptides, if more effective, could be used for any of the
5 purposes described herein.

Therefore, in addition to epitopes which are identical to the naturally occurring H-Y-specific epitopes, the present invention embraces epitopes which are substantially homologous with such epitopes, and therefore H-Y-specific in their own
10 right.

The term "substantially homologous", when used in connection with amino acid sequences, refers to sequences which are substantially identical to or similar in sequence with each other, giving rise to a homology in conformation and thus to
15 retention, to a useful degree, of one or more biological (including immunological) activities. The term is not intended to imply a common evolution of the sequences.

Substantially homologous peptide epitopes may be identified by a variety of techniques. It is known in the art that one may
20 synthesize all possible single substitution mutants of a known peptide epitope. Geysen, et al., Proc Nat. Acad. Sci. (USA), 81:3998-4002 (1984). For SPSVDKARAEI, there are only (19x11=) 209 such mutants. While the effects of different substitutions are not always additive, it is reasonable to expect that two
25 favorable or neutral single substitutions at different residue positions in the epitope can safely be combined in most cases.

One may also synthesize a family of related single or multiple substitution mutants, present the mixture to a cell line (such as HLA-B7+, H-Y- T2-B7 cells) capable of presenting
30 H-Y-specific CTL epitopes, and expose the cells to suitably restricted (e.g., HLA-B7) H-Y-specific CTLs. If the cells are lysed, the effective epitopes may be identified either by direct recovery from the T2 cells or by a progressive process of testing subsets of the effective peptide mixtures. Methods for
35 the preparation of degenerate peptides are described in Rutter, USP 5,010,175, Haughten, et al., Proc. Nat. Acad. Sci. (USA), 82:5131-35 (1985), Geysen, et al., Proc. Nat. Acad. Sci. (USA), 81:3998-4002 (1984); W086/06487; W086/00991.

In devising a multiple mutagenesis strategy, a person of ordinary skill would of course give weight to the single substitution mutant data in determining both which residues to vary and which amino acids (or classes of amino acids) are
5 suitable replacements.

The person of ordinary skill in the art, in determining which residues to vary, may also make comparisons of the sequences of the naturally processed MHC associated peptides, and may obtain 3D structures of the MHC: peptide: TCR complexes,
10 in order to identify residues involved in MHC or TCR binding. Such residues may either be left alone, or judiciously mutated in an attempt to alter MHC or TCR binding.

It is also possible to predict substantially homologous epitopes by taken into account studies of sequence variations
15 in families of naturally occurring homologous proteins. (In view of certain observed sequence homologies discussed in the Examples, proteins of myosin class I may be especially relevant.) Certain amino acid substitutions are more often tolerated than others, and these are often correlatable with
20 similar-ities in size, charge, etc. between the original amino acid and its replacement. Insertions or deletions of amino acids may also be made.

Conservative substitutions are herein defined as exchanges within one of the following five groups:

- 25 I. Small aliphatic, nonpolar or slightly polar residues:
Ala, Ser, Thr, Pro, Gly
- II. Polar, negatively charged residues: and
their amides
30 Asp, Asn, Glu, Gln
- III. Polar, positively charged residues:
His, Arg, Lys
- IV. Large, aliphatic, nonpolar residues:
Met, Leu, Ile, Val, Cys
- 35 V. Large, aromatic residues:
Phe, Tyr, Trp

Within the foregoing groups, the following substitutions are considered "highly conservative":

Asp/Glu
His/Arg/Lys
Phe/Tyr/Trp
Met/Leu/Ile/Val

5 Semi-conservative substitutions are defined to be exchanges between two of groups (I)-(V) above which are limited to supergroup (A), comprising (I), (II) and (III) above, or to supergroup (B), comprising (IV) and (V) above.

As discussed hereafter, a double substitution mutant (Ser3-
10 >Ala; Arg8->Gln) of the lead peptide was still able to sensitize T2-B7 cells for recognition, but had only 1/10,000th the potency of the lead peptide. However, since the purpose of the invention is not to achieve a strong immune response, but rather a muted one that serves to tolerize against the H-Y antigen,
15 such a mutant may well be useful, indeed, it could be more useful than the native epitope. In this regard, it is desirable to note that while the Ser->Ala mutation is a relatively conservative one (one small, aliphatic, uncharged a.a. for another), the Arg->Gln mutation is not. Further mutagenesis
20 experiments are necessary to determine whether the change in potency is attributable to the loss of H-bonding potential (by replacing Ser with Ala), the loss of the charge of the Arg, and/or a steric effect.

In the case of the aforementioned peptide, a possible
25 multiple mutagenesis strategy could be the following:
Parental Ser Pro Ser Val Asp Lys Ala Arg Ala Glu Leu

Replaced	Ala	Ala	Ala	Ile	Glu	Arg	Ser	Lys	Ser	Asp	Ile
by	Thr	Thr	Thr	Leu	Asn	His	Thr	His	Thr	Asn	Val
	Gly	Gly	Gly	Met	Gln				Gly	Gln	Met
30	Pro	Ser	Pro						Pro		

wherein each amino acid sequence in the parental sequence may either be retained, or replaced by one of the amino acids in the column below it, selected independently.

Substitutions are not limited to the genetically encoded,
35 or even the naturally occurring amino acids. When the epitope is prepared by peptide synthesis, the desired amino acid may be used directly. Alternatively, a genetically encoded amino acid

may be modified by reacting it with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. The following examples of chemical derivatives are provided by way of illustration and not by way
5 of limitation.

Aromatic amino acids may be replaced with D- or L-naphylalanine, D- or L-Phenylglycine, D- or L-2-thieneylalanine, D- or L-1-, 2-, 3- or 4-pyreneylalanine, D- or L-3-thieneylalanine, D- or L-(2-pyridinyl)-alanine, D- or
10 L-(3-pyridinyl)-alanine, D- or L-(2-pyrazinyl)-alanine, D- or L-(4-isopropyl)-phenylglycine, D-(trifluoromethyl)-phenylglycine, D-(trifluoromethyl)-phenylalanine, D-p-fluorophenylalanine, D- or L-p-biphenylphenylalanine, D- or L-p-methoxybiphenylphenylalanine, D- or L-2-indole-
15 (alkyl)alanines, and D- or L-alkylainines where alkyl may be substituted or unsubstituted methyl, ethyl, propyl, hexyl, butyl, pentyl, iso-propyl, iso-butyl, sec-isotyl, iso-pentyl, non-acidic amino acids, of C1-C20.

Acidic amino acids can be substituted with non-carboxylate
20 amino acids while maintaining a negative charge, and derivatives or analogs thereof, such as the non-limiting examples of (phosphono)-alanine, glycine, leucine, isoleucine, threonine, or serine; or sulfated (e.g., -SO₃H) threonine, serine, tyrosine.

Other substitutions may include unnatural hydroxylated amino
25 acids made by combining "alkyl" (as defined and exemplified herein) with any natural amino acid. Basic amino acids may be substituted with alkyl groups at any position of the naturally occurring amino acids lysine, arginine, ornithine, citrulline,
30 or (guanidino)-acetic acid, or other (guanidino)alkyl-acetic acids, where "alkyl" is define as above. Nitrile derivatives (e.g., containing the CN-moiety in place of COOH) may also be substituted for asparagine or glutamine, and methionine sulfoxide may be substituted for methionine. Methods of
35 preparation of such peptide derivatives are well known to one skilled in the art.

In addition, any amide linkage can be replaced by a

ketomethylene moiety, e.g. $(-C(=O)-CH_2-)$ for $(-C(=O)-NH-)$. Such derivatives are expected to have the property of increased stability to degradation by enzymes, and therefore possess advantages for the formulation of compounds which may have
5 increased in vivo half lives, as administered by oral, intravenous, intramuscular, intraperitoneal, topical, rectal, intraocular, or other routes.

In addition, any amino acid can be replaced by the same amino acid but of the opposite chirality. Thus, any amino acid
10 naturally occurring in the L-configuration (which may also be referred to as the R or S configuration, depending upon the structure of the chemical entity) may be replaced with an amino acid of the same chemical structural type, but of the opposite chirality, generally referred to as the D- amino acid but which
15 can additionally be referred to as the R- or the S-, depending upon its composition and chemical configuration. Such derivatives have the property of greatly increased stability to degradation by enzymes, and therefore are advantageous in the formulation of compounds which may have longer in vivo half
20 lives, when administered by oral, intravenous, intramuscular, intraperitoneal, topical, rectal, intraocular, or other routes.

Additional amino acid modifications of amino acids may include the following: Cysteiny l residues may be reacted with alpha-haloacetates (and corresponding amines), such as
25 2-chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteiny l residues may also be derivatized by reaction with compounds such as bromotrifluoroacetone, alpha-bromo-beta-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides,
30 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues may be derivatized by reaction with compounds such as diethylprocarbonate e.g., at pH 5.5-7.0
35 because this agent is relatively specific for the histidyl side chain, and para-bromophenacyl bromide may also be used; e.g., where the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues may be reacted with compounds such as succinic or other carboxylic acid anhydrides. Derivatization with these agents is expected to have the effect of reversing the charge of the lysinyl residues. Other suitable
5 reagents for derivatizing alpha-amino-containing residues include compounds such as imidoesters/e.g., as methyl picolinimide; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed
10 reaction with glyoxylate.

Arginyl residues may be modified by reaction with one or several conventional reagents; among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin according to known method steps. Derivatization of arginine residues
15 requires that the reaction be performed in alkaline conditions because of the high pKa of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues per se is
20 well-known, such as for introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. N-acetylimidazol and tetranitromethane may be used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

25 Carboxyl side groups (aspartyl or glutamyl) may be selectively modified by reaction with carbodiimides ($R'-N-C-N-R'$) such as 1-cyclohexyl-3-(2-morpholinyl- (4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues may be converted to
30 asparaginy and glutaminy residues by reaction with ammonium ions.

Glutaminy and asparaginy residues may be readily deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues may be deamidated under mildly
35 acidic conditions. Either form of these residues falls within the scope of the present invention.

Derivatization with bifunctional agents is useful for cross-linking the peptide to a water-insoluble support matrix

or to other macromolecular carriers, according to known method steps. Commonly used cross-linking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with
5 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimide yield
10 photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642;
15 4,229,537; and 4,330,440 (which are herein incorporated entirely by reference), may be employed for protein immobilization.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the alpha-amino groups of lysine,
20 arginine, and histidine side chains (Creighton, T.E., Proteins: Structure and Molecule Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, methylation of main chain amide residues (or substitution with N-methyl amino acids) and, in some instances, amidation of
25 the C-terminal carboxyl groups, according to known method steps. Glycosylation is also possible.

Derivatized moieties may improve the solubility, absorption, biological half life, and the like, or eliminate or attenuate any possible undesirable side effect of the molecule.
30 Moieties capable of mediating such effects are disclosed, for example, in Remington's Pharmaceutical Sciences, 16th ed., Mack Publishing Co., Easton, PA (1980).

Modifications are not limited to the side chains of the amino acids. One may also modify the peptidyl linkage itself,
35 e.g., -NRCO- (where R is alkyl or aryl), instead of -NHCO-, as in the so-called "peptoids".

It is also possible to construct and use so-called peptide mimetics whose conformation is similar to that of a peptide but

do not have a peptide-like molecular formula.

The H-Y-Specific Tolerogen

The H-Y-specific tolerogen of the present invention is a molecule corresponding to or otherwise comprising a H-Y-specific CTL epitope as previously described. The tolerogen may comprise one or more H-Y-specific CTL epitopes, which may be the same or different. If different, the epitopes may be chosen so that at least one epitope is effective in each of two or more restriction systems, e.g., HLA-B7 and HLA-A2. If the tolerogen comprises a plurality of such epitopes, they may be linked directly, or through a spacer of some kind, or by noncovalent means such as an avidin:biotin complex. The tolerogen may take any form that is capable of eliciting a H-Y-specific tolerizing immune response. The tolerogenic conjugate may also comprise moieties intended to diminish the immune response, such as certain cytokines.

A variety of non-(H-Y) specific tolerogens are known which may either be conjugated to the H-Y specific epitope to form a unitary tolerogen, and/or used as independent molecules (either in the same composition or in a separately administered composition administered as part of the medication schedule).

Mode of Production

The peptide portion of the tolerogens of the present invention may be produced by any conventional technique, including

- (a) nonbiological synthesis by sequential coupling of component amino acids,
- (b) production by recombinant DNA techniques in a suitable host cell, and
- (c) chemical or enzymatic modification of a sequence made by (a) or (b) above.

In view of the size of the epitopes, and the practicality of using the epitopic peptides directly as tolerogens, method (a) is preferred. However, if it becomes advantageous to produce peptides longer than about 50 amino acids, method (b) is preferred.

Gene Expression. The peptides disclosed herein may be produced, recombinantly, in a suitable host, such as bacteria from the genera *Bacillus*, *Escherichia*, *Salmonella*, *Erwinia*, and yeasts from the genera *Hansenula*, *Kluyveromyces*, *Pichia*,
5 *Rhinosporidium*, *Saccharomyces*, and *Schizosaccharomyces*, insect cells, or cultured mammalian cells such as COS-1, CHO and C127. The more preferred hosts are microorganisms of the species *Pichia pastoris*, *Bacillus subtilis*, *Bacillus brevis*,
10 *Saccharomyces cerevisiae*, *Escherichia coli* and *Yarrowia lipolytica*. Any promoter, regulatable or constitutive, which is functional in the host may be used to control gene expression.

Standard reference works setting forth the general principles of recombinant DNA technology include Watson, J.D.,
15 et al., Molecular Biology of the Gene, Volumes I and II, The Benjamin/Cummings Publishing Company, Inc., publisher, Menlo Park, CA (1987); Darnell, J.E., et al., Molecular Cell Biology, Scientific American Books, Inc., publisher, New York, N.Y. (1986); Lewin, B.M., Genes II, John Wiley & Sons, publishers,
20 New York, N.Y. (1985); Old, R.W., et al., Principles of Gene Manipulation: An Introduction to Genetic Engineering, 2d edition, University of California Press, publisher, Berkeley, CA (1981); Sambrook, J., et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring
25 Harbor, NY (1989); and Ausubel, et al., Current Protocols in Molecular Biology, Wiley Interscience, N.Y., (1987, 1992). These references are herein entirely incorporated by reference.

Chemical Peptide Synthesis. Chemical peptide synthesis is a rapidly evolving area in the art, and methods of solid phase
30 peptide synthesis are well-described in the following references, hereby entirely incorporated by reference: (Merrifield, B., J. Amer. Chem. Soc. 85:2149-2154 (1963); Merrifield, B., Science 232:341-347 (1986); Wade, J.D., et al., Biopolymers 25:S21-S37 (1986); Fields, G.B., Int. J. Polypeptide Prot. Res. 35:161 (1990); MilliGen Report Nos. 2 and
35 2a, Millipore Corporation, Bedford, MA, 1987) Ausubel, et al, supra, and Sambrook, et al, supra.

In general, as is known in the art, such methods involve blocking or protecting reactive functional groups, such as free amino, carboxyl and thio groups. After polypeptide bond formation, the protective groups are removed (or de-protect-ed).
5 Thus, the addition of each amino acid residue requires several reaction steps for protecting and deprotecting. Current methods utilize solid phase synthesis, wherein the C-terminal amino acid is covalently linked to an insoluble resin particle large enough to be separated from the fluid phase by filtration. Thus,
10 reactants are removed by washing the resin particles with appropriate solvents using an automated programmed machine. The completed polypeptide chain is cleaved from the resin by a reaction which does not affect polypeptide bonds.

In the more classical method, known as the "tBoc method,"
15 the amino group of the amino acid being added to the resin-bound C-terminal amino acid is blocked with tert-butyloxycarbonyl chloride (tBoc). This protected amino acid is reacted with the bound amino acid in the presence of the condensing agent dicyclohexylcarbodiimide, allowing its carboxyl group to form
20 a polypeptide bond the free amino group of the bound amino acid. The amino-blocking group is then removed by acidification with trifluoroacetic acid (TFA); it subsequently decomposes into gaseous carbon dioxide and isobutylene. These steps are repeated cyclically for each additional amino acid residue. A
25 more vigorous treatment with hydrogen fluoride (HF) or trifluoro-methanesulfonyl derivatives is common at the end of the synthesis to cleave the benzyl-derived side chain protecting groups and the polypeptide-resin bond.

More recently, the preferred "Fmoc" technique has been
30 introduced as an alternative synthetic approach, offering milder reaction conditions, simpler activation procedures and compatibility with continuous flow techniques. This method was used, e.g., to prepare the peptide sequences disclosed in the present application. Here, the α -amino group is protected by
35 the base labile 9-fluorenylmethoxycarbonyl (Fmoc) group. The benzyl side chain protecting groups are replaced by the more acid labile t-butyl derivatives. Repetitive acid treatments are replaced by deprotection with mild base solutions, e.g., 20%

piperidine in dimethyl-formamide (DMF), and the final HF cleavage treatment is eliminated. A TFA solution is used instead to cleave side chain protecting groups and the peptide resin linkage simultaneously.

5 At least three different peptide-resin linkage agents can be used: substituted benzyl alcohol derivatives that can be cleaved with 95% TFA to produce a peptide acid, methanolic ammonia to produce a peptide amide, or 1% TFA to produce a protected peptide which can then be used in fragment
10 condensation procedures, as described by Atherton, E., et al., J. Chem. Soc. Perkin Trans. 1:538-546 (1981) and Sheppard, R.C., et al., Int. J. Polypeptide Prot. Res. 20:451-454 (1982). Furthermore, highly reactive Fmoc amino acids are available as pentafluorophenyl esters or dihydro-oxobenzotriazine esters
15 derivatives, saving the step of activation used in the tBoc method.

Pharmaceutical Use

The pharmaceutical compositions of the present invention may be used to protect mammals against allogeneic immune
20 responses to a foreign H-Y antigen. One or more such compositions will be used as part of a tolerogenically effective medication schedule to achieve such protection.

By the term "mammal" is meant an individual belonging to the class Mammalia, which, of course, includes humans. The
25 invention is particularly useful in the treatment of human subjects, although it is intended for veterinary uses as well.

Among mammals, the subject preferably belongs to the order Primata (humans, apes, and monkeys), Artiodactyla (e.g., cows, pigs, sheep, goats, horses), Rodenta (e.g., rabbits, mice,
30 rats), or Carnivora (e.g., cats, dogs). Primates are especially preferred.

By the term "non-human primate" is intended any member of the suborder Anthroidea except for the family Hominidae. Such non-human primates include the superfamily Ceboidea, family
35 Cebidae (the New World monkeys including the capuchins, howlers, spider monkeys and squirrel monkeys) and family Callithricidae (including the marmosets); the superfamily Cercopithecoidea,

family Cercopithecidae (including the macaques, mandrills, baboons, proboscis monkeys, mona monkeys, and the sacred hunaman monkeys of India); and superfamily Hominoidea, family Pongidae (including gibbons, orangutans, gorillas, and chimpanzees). The
5 rhesus monkey is one member of the macaques.

The term "protection", as used herein, is intended to include "prevention," "suppression" and "treatment." "Prevention" involves administration of the protective composition prior to the induction of the disease.
10 "Suppression" involves administration of the composition prior to the clinical appearance of the disease. "Treatment" involves administration of the protective composition after the appearance of the disease.

It will be understood that in human and veterinary
15 medicine, it is not always possible to distinguish between "preventing" and "suppressing" since the ultimate inductive event or events may be unknown, latent, or the patient is not ascertained until well after the occurrence of the event or events. Therefore, it is common to use the term "prophylaxis"
20 as distinct from "treatment" to encompass both "preventing" and "suppressing" as defined herein. The term "protection," as used herein, is meant to include "prophylaxis."

It should also be understood that to be useful, the protection provided need not be absolute, provided that it is
25 sufficient to carry clinical value. An agent which provides protection to a lesser degree than do competitive agents may still be of value if the other agents are ineffective for a particular individual, if it can be used in combination with other agents to enhance the overall level of protection, or if
30 it is safer than competitive agents.

The form of administration may be systemic or topical. For example, administration of such a composition may be by various parenteral routes such as subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal,
35 transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. Parenteral administration can be by bolus injection or by gradual perfusion over time.

A typical regimen (medication schedule) comprises administration of an effective amount of the tolerogen, administered over a period ranging from a single dose, to dosing over a period of hours, days, weeks, months, or years.

5 The doses may be equal in size, or different. If different, they may increase, or decrease, in a regular manner, or otherwise. The physician will usually exercise medical discretion and decrease or increase the dose after consideration of the effect of the initial dose(s) on the patient.

10 Similarly, the interval between doses may be constant, or it may be increased or decreased.

It is understood that the suitable dosage of a tolerogen of the present invention will be dependent upon the age, sex, health, and weight of the recipient, kind of concurrent
15 treatment, if any, frequency of treatment, and the nature of the effect desired. However, the most preferred dosage can be tailored to the individual subject, as is understood and determinable by one of skill in the art, without undue experimentation. This will typically involve adjustment of a
20 standard dose, e.g., reduction of the dose if the patient has a low body weight.

Prior to use in humans, a drug will first be evaluated for safety and efficacy in laboratory animals. In human clinical studies, one would begin with a dose expected to be safe in
25 humans, based on the preclinical data for the drug in question, and on customary doses for analogous drugs (if any). If this dose is effective, the dosage may be decreased, to determine the minimum effective dose, if desired. If this dose is ineffective, it will be cautiously increased, with the patients
30 monitored for signs of side effects. See, e.g., Berkow, et al., eds., The Merck Manual, 15th edition, Merck and Co., Rahway, N.J., 1987; Goodman, et al., eds., Goodman and Gilman's The Pharmacological Basis of Therapeutics, 8th edition, Pergamon Press, Inc., Elmsford, N.Y., (1990); Avery's Drug Treatment: Principles and Practice of Clinical Pharmacology and
35 Therapeutics, 3rd edition, ADIS Press, LTD., Williams and Wilkins, Baltimore, MD. (1987), Ebadi, Pharmacology, Little, Brown and Co., Boston, (1985), which references and references

cited therein, are entirely incorporated herein by reference.

The total dose required for each treatment may be administered by multiple doses or in a single dose. The tolerogen may be administered alone or in conjunction with other
5 therapeutics directed to the disease or directed to other symptoms thereof.

The appropriate dosage form will depend on the disease, the tolerogen, and the mode of administration; possibilities include tablets, capsules, lozenges, dental pastes, suppositories,
10 inhalants, solutions, ointments and parenteral depots. See, e.g., Berker, supra, Goodman, supra, Avery, supra and Ebadi, supra, which are entirely incorporated herein by reference, including all references cited therein.

It is expected that each composition will include .001-
15 1,000 micrograms, more typically .1-1000 μ g, of the peptide epitope, but applicants are not to be considered to be bound to this range. It is noted that in WO90/08162, the therapeutically effective concentration was expected to be on the order of 100 micromoles of the "replacing peptide".

20 In addition to at least one tolerogen as described herein, a pharmaceutical composition may contain suitable pharmaceutically acceptable carriers, such as excipients, carriers and/or auxiliaries which facilitate processing of the active compounds into preparations which can be used
25 pharmaceutically. See, e.g., Berker, supra, Goodman, supra, Avery, supra and Ebadi, supra, which are entirely incorporated herein by reference.

The composition of the present invention may also include any compound used in the prevention or treatment of graft
30 rejection or graft-versus-host disease, such as an immunosuppressive agent, or a less specific tolerizing agent.

For discussion of immunosuppressive agents, tolerogens, tolerogenic conjugates, and/or tolerogenizing methods, see Maiti, US 5,358,710; Conrad, US 5,276,013, Cohen, US 5,114,844;
35 Burnierm US 5,061,786; Cianciolo, US 4,822,606; Lee, US 4,430,260; US 4,261,973; WO95/10301; WO94/07516; WO94/05323; WO93/12145; WO92/21981; WO91/10426; DD278,721; WO91/08773; EP 243,044; US 4,650,675; Scheper, et al., Arch. Toxicol. Suppl.

16: 63-70 (1994); Abbas et al., Semin. Immunol., 5: 441-7 (1993); Adorini, Clin. Exp. Rheumatol., 11 Suppl. 8: S41-4 (1993); Lobo-Yeo, et al., Clin. Exp. Rheumatol., 11 Suppl. 8: S17-21 (1993); Olive et al., Crit. Rev. Ther. Drug Carrier Syst, 10: 29-63
5 (1993); Rayner et al., Immunol. Ser., 59: 359-76 (1993); Lo, Curr. Opin. Immunol., 4: 711-5 (1992); Dua et al., Curr. Eye Res., 11 Suppl. 59-65 (1992); Sehon, Adv. Exp. Med. Biol., 303: 199-206 (1991); Sehon, Int. Arch. Allergy Appl. Immunol., 94: 11-20 (1991); Welsh, Pediatr. Nephrol., 5: 622-9 (1991); Davies,
10 et al., Transplant Proc., 23: 2248-9 (1991); Sehon, Adv. Exp. Med. Biol., 251: 341-51 (1989); Borel, Concepts Immunopathol, 7: 145-61 (1989); ; Makay et al., Postgrad. Med. J., 64: 522-30 (1988); Miele, et al., Endocr. Rev., 8: 474-90 (1987); Scott, Crit. Rev. Immunol., 5: 1-25 (1984); Sehon, Ann. N.Y. Acad.
15 Sci., 392: 55-70 (1982); Sehon, Prog. Allergy, 32: 161-202 (1982); Fidler, J. Exp. Med., 3: 491-506 (1979); Schneider, Pathol. Microbiol., 42: 254-66 (1975).

H-Y Specific Diagnostic Agents

An H-Y specific diagnostic agent is (1) a molecule which
20 is or which comprises an H-Y specific epitope as previously defined, and which is labeled, immobilized, or otherwise rendered suitable for diagnostic use, or (2) an antibody which
specifically binds such an H-Y specific epitope, and which is
labeled, immobilized, or otherwise rendered suitable for
25 diagnostic use.

Diagnostic Uses and Compositions

The diagnostic agents of the present invention may be used to screen a sample for the presence of an antigen with the same epitope, or with a different but cross-reactive epitope, or for
30 the presence of CTLs which specifically recognize the corresponding epitopes. In particular, the assay may be used to determine (1) whether an intended recipient is likely to suffer, in the absence of treatment, a severe graft rejection or graft-vs.-host disease as a result of the immune response to
35 a particular H-Y CTL epitope; and (2) whether, after transplantation of bone marrow from a female donor to a male

recipient, the post-transplant hematopoietic compartment of the bone marrow include a significant number of H-Y+ (i.e., native) cells.

The sample will normally be a biological fluid, such as
5 blood, urine, lymphatic fluid, amniotic fluid, semen, saliva, tears, milk, or cerebrospinal fluid, or a fraction or derivative thereof, or a biological tissue, in the form of, e.g., a tissue section or homogenate. The preferred sample is blood, or a fraction or derivative thereof.

10 Assays may be divided into two basic types, hetero-geneous and homogeneous. In heterogeneous assays, the interaction between the affinity molecule and the analyte does not affect the label, hence, to determine the amount or presence of analyte, bound label must be separated from free label. In
15 homogeneous assays, the interaction does affect the activity of the label, and therefore analyte levels can be deduced without the need for a separation step.

Assays may also be divided into competitive and non-competitive formats. In the competitive format, the analyte
20 competes with a labeled analyte analogue for binding to a binding partner. In a common noncompetitive format called a sandwich assay, the analyte is first bound by a capture reagent, and then by a tag reagent.

In order to detect the presence, or measure the amount, of
25 an analyte, the assay must provide for a signal producing system (SPS) in which there is a detectable difference in the signal produced, depending on whether the analyte is present or absent (or, in a quantitative assay, on the amount of the analyte). The detectable signal may be one which is visually detectable,
30 or one detectable only with instruments. Possible signals include production of colored or luminescent products, alteration of the characteristics (including amplitude or polarization) of absorption or emission of radiation by an assay component or product, and precipitation or agglutination of a
35 component or product. The term "signal" is intended to include the discontinuance of an existing signal, or a change in the rate of change of an observable parameter, rather than a change in its absolute value. The signal may be monitored manually or

automatically.

The component of the signal producing system which is most intimately associated with the diagnostic reagent is called the "label". A label may be, e.g., a radioisotope, a fluorophore, 5 an enzyme, a co-enzyme, an enzyme substrate, an electron-dense compound, an agglutinable particle.

The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography. Isotopes which are particularly useful for the 10 purpose of the present invention are ^3H , ^{125}I , ^{131}I , ^{35}S , ^{14}C , and, preferably, ^{125}I .

It is also possible to label a compound with a fluorescent compound. When the fluorescently labeled anti-body is exposed to light of the proper wave length, its presence can then be 15 detected due to fluorescence. Among the most commonly used fluorescent labelling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, α -phthaldehyde and fluorescamine.

Alternatively, fluorescence-emitting metals such as ^{125}Eu , 20 or others of the lanthanide series, may be attached to the binding protein using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA).

The diagnostic agents also can be detectably labeled by 25 coupling to a chemiluminescent compound. The presence of the chemiluminescently labeled antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isolumino, 30 theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the agents. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein 35 increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bio-luminescent compounds for purposes of labeling are luciferin, luciferase and

aequorin.

Enzyme labels, such as horseradish peroxidase, alkaline phosphatase, malate dehydrogenase, staphylococcal nuclease, δ -V-steroid isomerase, yeast alcohol dehydrogenase, α -glycero phosphate dehydrogenase, triose phosphate isomerase, asparaginase, glucose oxidase, β -galactosidase, ribonuclease, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholine esterase, are preferred. When an enzyme label is used, the signal producing system must also include a substrate for the enzyme. If the enzymatic reaction product is not itself detectable, the SPS will include one or more additional reactants so that a detectable product appears.

A label may be conjugated, directly or indirectly (e.g., through a labeled antibody), covalently (e.g., with SPDP) or noncovalently, to the epitope, to produce a diagnostic reagent. Similarly, the epitope may be conjugated to a solid phase support to form a solid phase ("capture") diagnostic reagent. Suitable supports include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to its target. Thus the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc.

30 Sex Selection

The peptides of the present invention may be used to generate cytotoxic lymphocytes (CTL) or monoclonal antibodies (mAb) that could be used to select for male and female sperm cells, allowing for the selection of the sex of a resulting embryo after fertilization. We and others have developed CTL that recognize peptides presented by human class I MHC molecules using mice that express the human class I molecules in transgenic form. This is accomplished by immunization of the

mice with the peptide, and removal and culture of T cells from the mice in the presence of additional peptide and interleukin 2. Stable long term T cell lines and clones can be obtained from the spleen cell culture and used as reagents. Similarly, these animals may be used to isolate antibodies directed against the complex of the peptide with an MHC molecule. Antibodies with this type of specificity have been previously reported in the literature. In the context of the present invention, we would produce CTL and mAb directed against the complex of (1) a class I MHC molecule and (2) an H-Y and/or SMCY-epitope bearing peptide.

Such CTL or mAb could be used in either of two methods for selecting sperm of the appropriate sex. In the negative selection method, CTL would be added to a population of sperm. The CTL would directly kill any sperm cells expressing the peptide-MHC complex for which the CTL was specific. For example, CTL directed against a male specific H-Y peptide and/or SMCY peptide would kill all male (Y-chromosome bearing) sperm, leaving the female (non-Y-chromosome bearing) sperm. A similar end could be achieved using a complement fixing mAb directed against the same antigen, after addition of complement. In the second method, positive selection, an antibody of the appropriate specificity would be coupled to a plastic dish or a magnetic bead, allowing the binding and separation of sperm bearing the appropriate antigen. In either method, the sperm selected would be used for fertilization of eggs, followed by reimplantation of the eggs into a suitable host.

EXAMPLES

Cytotoxicity Assays

Cell-mediated killing was determined in vitro using a 4-h chromium release assay. 51Cr-labeled target cells were plated at 2×10^3 cells/well in triplicate on 96-well V-bottom plates (Costar, Cambridge, MA) with varying numbers of effector cells in a final volume of 250 microl. Wells containing either culture medium and target cells only or 1 M HCl and target cells served as background 51Cr release controls and total release controls, respectively. The plates were centrifuged at 100 x

g for 3 min and incubated at 37°C in 5% CO₂ for 4 h. The plates were again centrifuged, and 0.20 ml of medium from each well was removed for counting in a gamma counter. The cytotoxic index was calculated as:

$$\frac{\text{Cpm (experimental)} - \text{cpm (background)}}{\text{Cpm (total release)} - \text{cpm (background)}} \times 100\%$$

Lytic units were calculated for several of the cytotoxicity assays, using a software package prepared by the National Cancer Institute (Bethesda, MD), which solves for the equation $y = A \times [1 - \exp(-kx)]$, where x is the E:T ratio, y is the cytotoxic index, A is the curve maximum, and k is a constant used to calculate the slope of the best fit line. For the purposes of this study, a lytic unit was defined as the number of effector cells needed to mediate 30% lysis of target cells. The number of lytic units was calculated per 10^5 effector cells (LU30 per 10^5 cells).

Isolation of H-Y Peptides

To isolate endogenously processed H-Y peptides, HLA-B7 molecules were purified by affinity chromatography from the H-Y positive, B lymphoblastoid cell line, JY (14). The associated peptides were extracted in acid and separated from high molecular weight material by ultrafiltration as previously described (15), and subsequently fractionated by reverse-phase high-performance liquid chromatography (HPLC) (11). Aliquots of each fraction were incubated with HLA-B7 positive, H-Y negative T2-B7 target cells in order to assay for the ability to reconstitute the epitope recognized by an HLA-B7-restricted, H-Y specific CTL clone, 5W4 (16). A single peak of reconstituting activity was observed (Fig. 1A, fraction 28 and 29), which was rechromatographed using a different organic modifier. Although a single active peak of reconstituting activity was also observed from this separation (Fig 1B, fraction 14, 15 and 16), it still contained more than 100 distinct peptide species, as assessed by electrospray ionization tandem mass spectrometry.

To identify the active H-Y peptide in this mixture, we applied each active fraction separately to a microcapillary HPLC

column and split the effluent following the separation (11):
Four-fifths of the effluent was directed into the mass
spectrometer for analysis, while one-fifth was simultaneously
directed into a 96-well microtiter plate for a subsequent
5 epitope reconstitution assay. The amount of the H-Y sensitizing
activity in each well was correlated to signals observed in the
mass spectrum, and therefore to the abundance of different
peptide species. By comparing the profile of H-Y activity and
the ion abundance data (Fig. 2), we were able to identify an
10 $(M+3H)^{+3}$ ion at a mass-to-charge ratio (m/z) of 391 (neutral
molecular mass =1171), whose abundance correlated with the
amount of H-Y epitope reconstituting activity. Further
confirmation of the importance of peptide 1171 was provided by
the demonstration that a peptide with an identical mass and
15 collision-activated dissociation (CAD) spectrum was also present
in HLA-B7 associated peptides extracted from a second H-Y
positive B lymphoblastoid line, DM, but absent from a
spontaneous H-Y antigen loss variant of this cell, DM(-) (17).

Assignment of a complete amino acid sequence to the 1171
20 peptide from the CAD mass spectrum recorded at the 20 fmol level
proved difficult due to the absence of high mass fragment ions
containing the amine terminus (b-type ions). A series of single
and / or doubly charged fragment ions containing the carboxyl
terminus (y-type ions) identified the C-terminal residue as
25 either L or I and the first six amino acids as SPSVDK. The
difference in molecular mass between this partial sequence and
that of the full length peptide suggested the presence of four
additional residues, for a total length of 11. Since the
candidate peptide existed exclusively in the gas phase as an
30 $(M+3H)^{+3}$ ion, and underwent mass shifts of 42 and 84 Da on
conversion to the corresponding methyl ester and acetylated
derivative, respectively, two of the remaining residues were
assigned as R and either D or E. Only two combinations of four
residues (AREA and GRDV) meet the above criteria and satisfy the
35 missing mass of 427 Da. CAD spectra recorded on synthetic
peptides suggested that R could not be located at either
position 7 or 10. Data bases were searched for proteins

containing peptides with these characteristics, and a sequence consistent at 9 out of 11 positions was found in residues 909-919 of the protein encoded by a gene called *XE169* or *SMCX* (18), which is located on the X chromosome. A homolog of *SMCX*, called
5 *SMCY*, is located on the Y chromosome (19). This protein (20) contains a sequence (residues 902-912) that is consistent at 11 out of 11 positions, and has the expected mass of 1171 Da. A CAD mass spectrum recorded on the naturally processed material after conversion of the R residue to ornithine confirmed that
10 its sequence was identical to that found in the *SMCY* protein (Fig. 3).

Sensitization and Affinity Assays

A synthetic peptide corresponding to the 11 residue *SMCY* sequence (SPSVDKARAEI) was found to sensitize T2-B7 cells for
15 recognition by the H-Y specific CTL clone. Half-maximal lysis was achieved at a peptide concentration of 10 pM (Fig. 4). The corresponding peptide derived from the sequence of the X chromosomal homolog, *SMCX*, has substitutions of A for S at position 3 and Q for R at position 8. Although this peptide
20 also was able to sensitize T2-B7 cells for recognition, comparable levels of killing were only achieved by using a 10,000-fold higher peptide concentration. Binding studies showed that the concentration of the *SMCY* peptide that inhibited the binding of an iodinated standard peptide to purified HLA-B7
25 by 50% (IC_{50}) was 34 nM, while the IC_{50} for the *SMCX* peptide was 140 nM (Fig. 5). Thus, the significant difference in the ability of the *SMCY* and *SMCX* peptides to sensitize targets for T cell recognition is almost entirely due to the fine specificity of the T cell receptor, rather than to differences
30 in MHC binding affinities. The *SMCX* peptide is also present in naturally processed peptide extracts of HLA-B7, although its abundance is only 25% of that of the *SMCY* peptide (17). Based on all of this information, we conclude that the peptide epitope representing the HLA-B7 restricted H-Y antigen is derived from
35 the protein encoded by *SMCY*. However, it is possible that the H-Y antigen is a not previously identified homologue of *SMCY* which happens to have the aforementioned peptide sequence in common with *SMCY*.

One interesting issue is whether the H-Y epitope peptides presented by other MHC molecules will also be derived from SMCY. SMCY and SMCX are 85% identical at the amino acid sequence level, and the SMCX gene is expressed ubiquitously from both the active and the inactive X chromosomes in both mice and human (18,22). Therefore, self-tolerance to SMCX will limit the number of SMCY peptides that could give rise to H-Y epitopes in association with different MHC molecules. On the other hand, SMCY contains almost 1500 residues, and the over 200 amino acid sequence differences between it and SMCX are scattered relatively uniformly throughout its length. Thus, there is the potential to generate a large number of distinct SMCY-specific peptides as H-Y epitopes. It is still an open question whether the H-Y epitope peptides presented by other MHC molecules are also derived from SMCY. Genetic mapping of the mouse Y chromosome has suggested at least two and up to five distinct loci encoding H-Y antigens (23). Interestingly, a murine H-Y epitope restricted by H-2K^k has also been shown to be derived from the murine Smcy protein (24). The demonstration that two H-Y epitopes from either mouse or human are derived from the same protein makes SMCY the prime target in searching other H-Y epitopes.

The identification of the protein that gives rise to an H-Y antigen culminates 40 years of uncertainty regarding its origin. However, the function of SMCY, as well as the homologous SMCX, remains unclear. Both proteins share significant sequence homology to retinoblastoma binding protein 2, which has been suggested to be a transcription factor (25). Nonetheless, this and other H-Y specific peptides are candidates for immunomodulatory approaches in bone marrow transplantation. They may also form the basis for genetic probes to be used for prenatal diagnosis in sex-linked congenital abnormalities, as well as for investigating minimal residual disease and chimerism.

All references cited anywhere in this specifications, whether patents, patent applications, or nonpatent publications, are hereby incorporated by reference.

References

1. B. Loveland, E. Simpson, *Immunol. Today* 7, 223 (1986).
2. E. J. Eichwald, C. R. Silmsler, *Transplant. Bull.* 2, 148 (1955); R. E. Billingham, W. K. Silvers, *J. Immunol.* 85, 14 (1960).
3. E. Goulmy, A. Termijtelen, B. A. Bradley, J. J. van Rood, *Lancet* 2, 1206 (1976); E. Goulmy, A. Termijtelen, B. A. Bradley, J. J. van Rood, *Nature* 266, 544 (1977).
4. P. J. Voogt et al, *Lancet* 335, 131 (1990).
5. *Bone Marrow Transplantation* 4, 221 (1989).
6. M. M. Bortin, *Transplant. Proc.* 19, 2655 (1987).
7. M. de Bueger, A. Bakker, J. J. van Rood, F. Van der Woude, E. Goulmy, *J. Immunol.* 149, 1788 (1992); D. van der Harst et al, *Blood* 83, 1060 (1994).
8. E. Goulmy, in *Transplantation Reviews*, Vol. 2, P. J. Morris and N. C. Tilney, Eds. (Saunders, Philadelphia, 1988), p. 29.
9. R. D. Gordon, E. Simpson, L. E. Samelson, *J. Exp. Med.* 142, 1108 (1975).
10. O. Rotzschke, K. Falk, H. J. Wallny, S. Faath, H. G. fRammensee, *Science* 249, 283 (1990).
11. A. L. Cox et al, *Science* 264, 716 (1994).
12. R. A. Henderson et al, *Proc. Natl. Acad. Sci. USA* 90, 10275 (1993).
13. J. M. M. den Haan et al, *Science* 268, 1476 (1995).
14. M. J. Turner et al, *J. Biol. Chem.* 250, 4512 (1975); P. Parham, B. N. Alpert, H. T. Orr, J. L. Strominger, *J. Biol. Chem.* 252, 7555 (1977).
15. D.F. Hunt et al, *Science* 255, 1261 (1992); E. L. Huczko et al, *J. Immunol.* 151, 2572 (1993).
16. E. Goulmy, J. D. Hamilton, B. A. Bradley, *J. Exp. Med.* 149, 545 (1979).
17. L. R. Meadows, W. Wang, N. E. Sherman, J. M. den Haan, unpublished results.
18. J. Wu et al, *Human Molecular Genetics* 3, 153 (1994); A. I. Agulnik et al, *Human Molecular Genetics* 3, 879 (1994).
19. A. I. Agulnik, M. J. Mitchell, J. L. Lerner, D. R. Woods, C. E. Bishop, *Human Molecular Genetics* 3, 873 (1994).

20. A. I. Agulnik, C. E. Bishop, unpublished results.
21. M. A. Cantrell, J. S. Bogan, E. Simpson, J. N. Bicknell,
E. Goulmy, et al, *Genomics* **13**, 1255 (1992).
22. J. Wu et al, *Nature Genetics* **7**, 491 (1994).
- 5 23. T. R. King et al, *Genomics* **24**, 159 (1994)
24. D. M. Scott et al, unpublished results.
25. A. R. Fattaey et al, *Oncogene* **8**, 3149 (1993).
26. J. Ruppert et al, *Cell* **74**, 929 (1993); Y. Chen et al, *J.*
Immunol. **152**, 2874 (1994); A. Sette et al, *J. Immunol.*
10 **153**, 5586 (1994).

We hereby claim:

1. A method of protecting a mammal against an undesired allogeneic immune response to an H-Y antigen which comprises administering to said mammal, according to a tolerogenically
5 effective medication schedule, one or more doses of an H-Y specific tolerogen.
2. The method of claim 1 in which the mammal is a human.
3. The method of claim 1 wherein the mammal is H-Y- and is protected from an immune response to an H-Y+ transplanted organ.
- 10 4. The method of claim 1 wherein the mammal is H-Y+ and is protected from an immune response mediated by H-Y- immunocytes in an organ transplanted from an H-Y- individual.
5. The method of claim 1 wherein the immune response is attributable to the transfusion of blood or blood components.
- 15 6. The method of claim 1 wherein the immune response is attributable to maternal recognition of fetal H-Y antigen.
7. The method of claim 1 wherein said tolerogen comprises an H-Y specific epitope which is identical to, or otherwise at least substantially homologous with, SPSVDKARAEEL.
- 20 8. The method of claim 1 wherein said tolerogen comprises an H-Y specific epitope which is identical to, or otherwise at least substantially homologous with, SPAVDKAQAEL.
9. The method of claim 1 wherein said tolerogen comprises an HLA-B restricted epitope.
- 25 10. The method of claim 9 wherein said epitope is HLA-B7 restricted.
11. The method of claim 10 wherein said epitope is recognized by the cell line 5W4.
12. A non-naturally occurring H-Y specific tolerogen.

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FIG. 1A

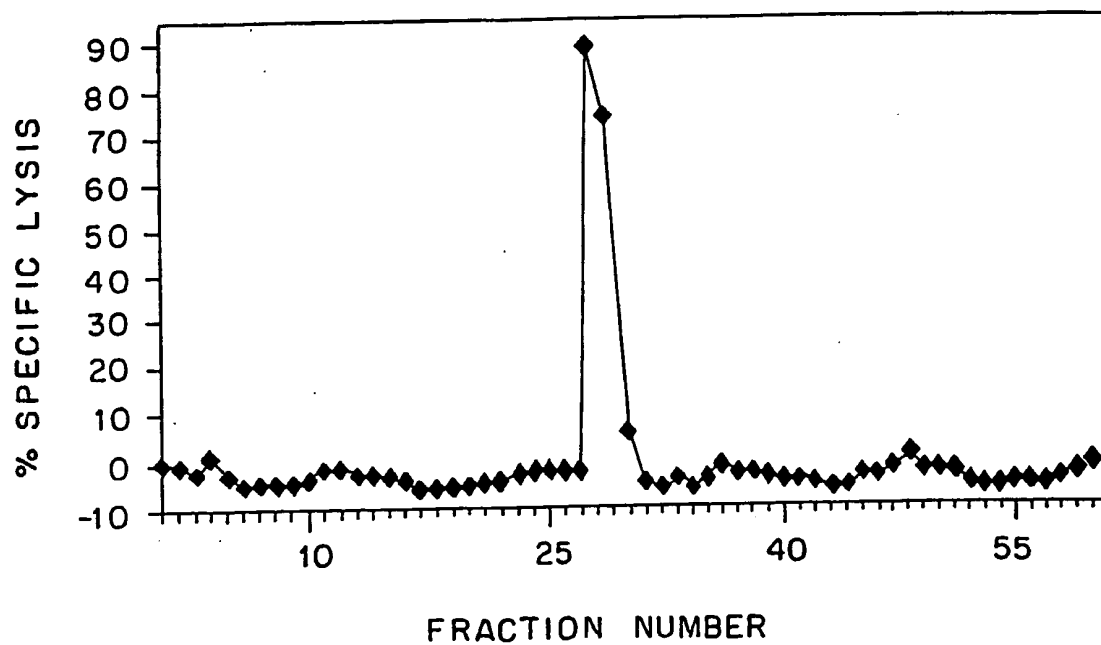
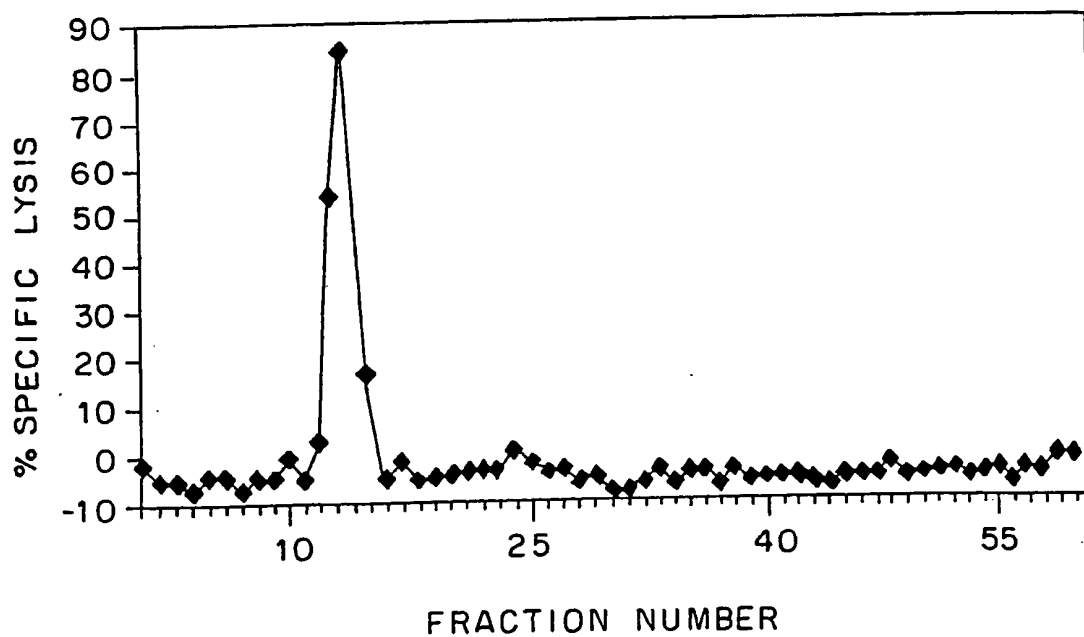


FIG. 1B



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FIG. 2

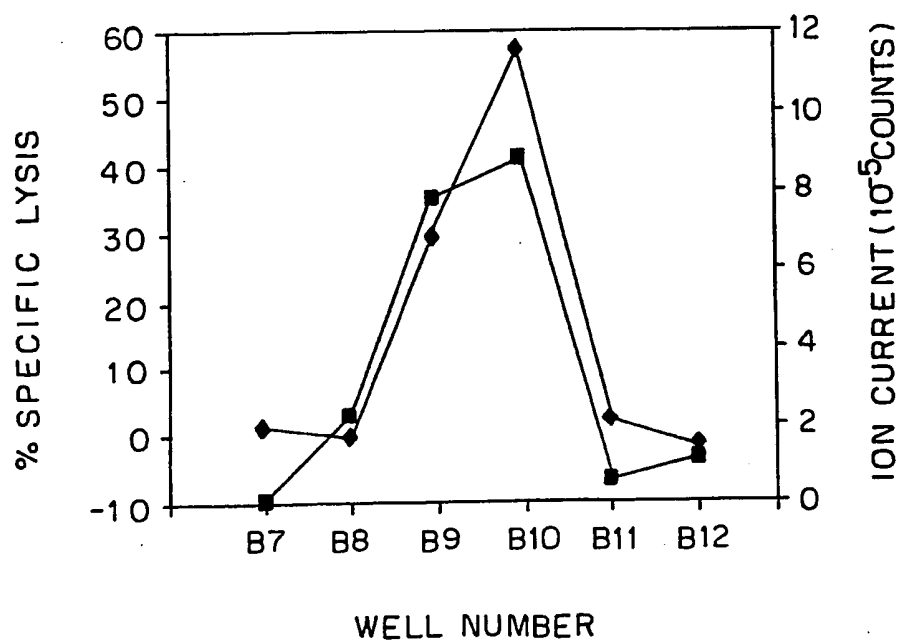
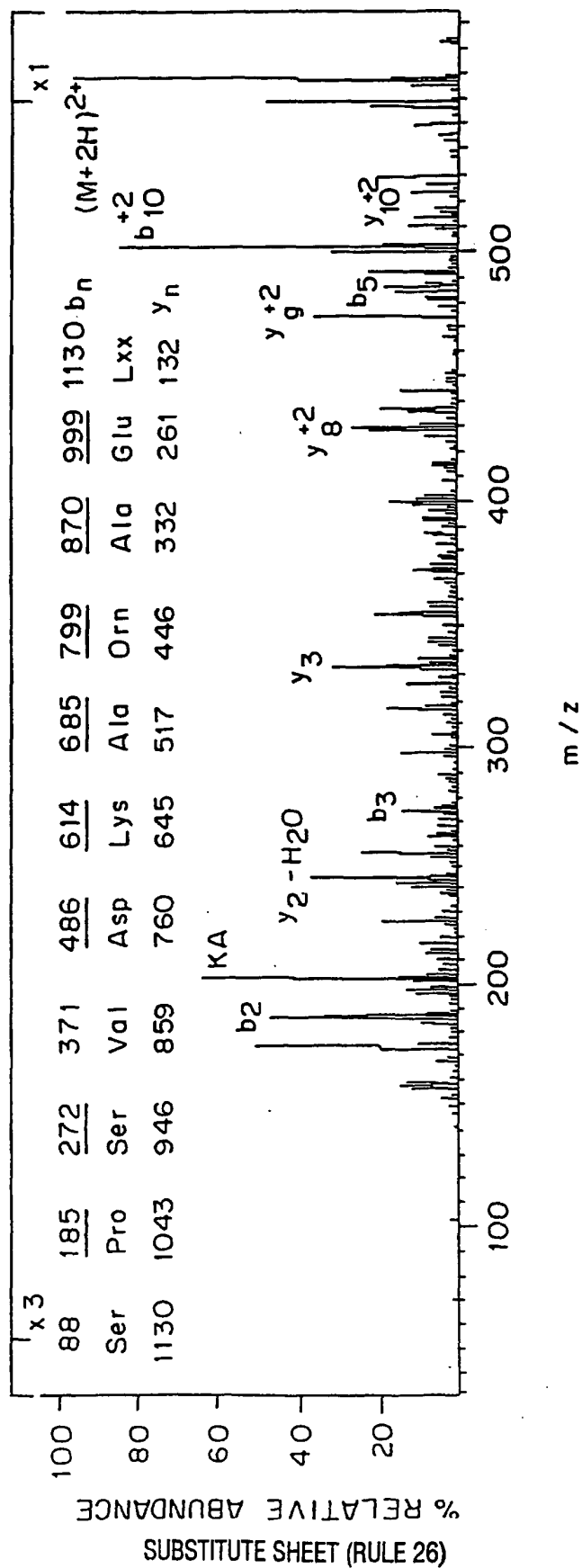
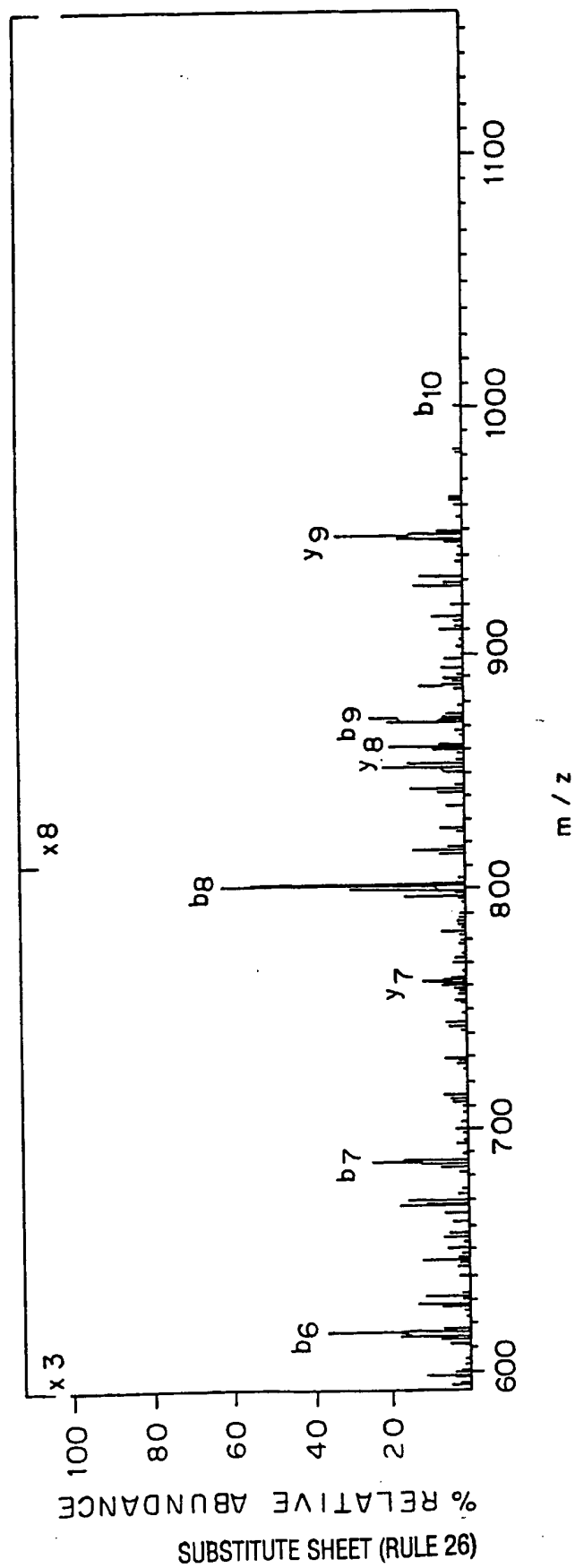


FIG. 3A



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FIG. 3B



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FIG. 4

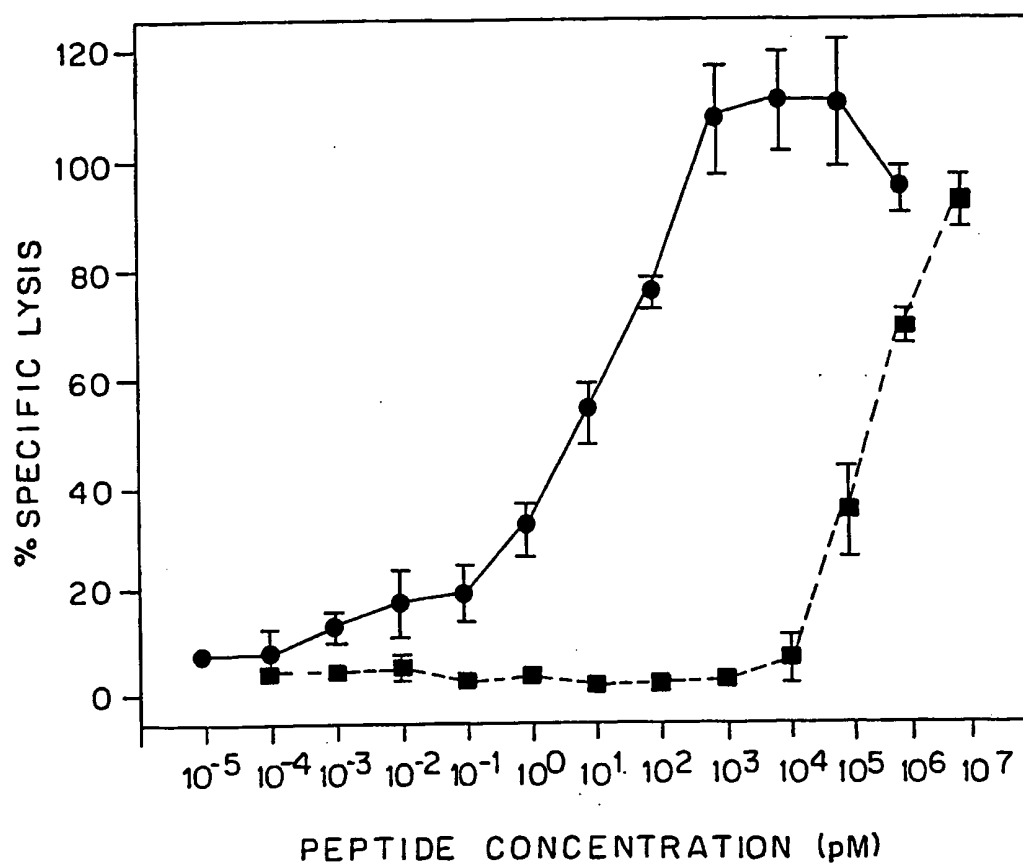
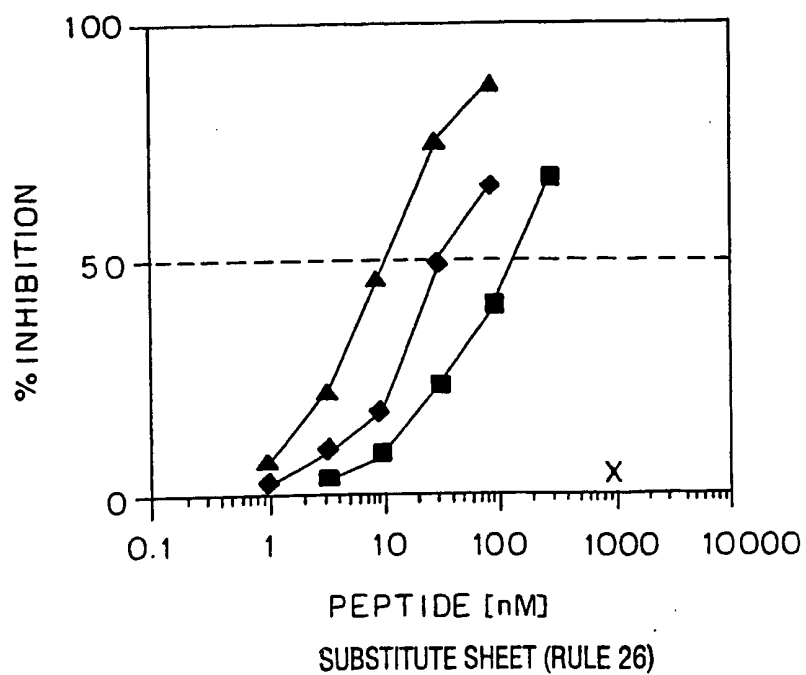


FIG. 5



INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/12351

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 A61K38/17 A61K39/00 C07K14/74

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GENOMICS, vol. 24, 1994, pages 159-168, XP000608200 KING ET AL: "DELETION MAPPING BY IMMUNOSELECTION AGAINST THE H-Y HISTOCOMPATIBILITY ANTIGEN FURTHER RESOLVES THE SXRA REGION OF THE MOUSE Y CHROMOSOME AND REVEALS COMPLEXITY OF THE HYA LOCUS" cited in the application ---	1-12
A	HUMAN MOLECULAR GENETICS, vol. 3, no. 6, 1994, pages 879-884, XP002020886 AGULNIK ET AL: "A NOVEL X GENE WITH A WIDELY TRANSCRIBED Y-LINKED HOMOLOGUE ESCAPES X-INACTIVATION IN MOUSE AND HUMAN" cited in the application ---	1-12
-/--		

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

Date of the actual completion of the international search

11 December 1996

Date of mailing of the international search report

10.01.1997

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Sitch, W

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/12351

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCIENCE, vol. 268, 9 June 1995, pages 1476-1480, XP002020887 DEN HAAN ET AL: "IDENTIFICATION OF A GRAFT VERSUS HOST DISEASE-ASSOCIATED HUMAN MINOR HISTOCOMPATIBILITY ANTIGEN" cited in the application ---	1-12
A	PATENT ABSTRACTS OF JAPAN vol. 11, no. 366 (P-641), 28 November 1987 & JP,A,62 138760 (OSAKA OXYGEN IND LTD), 22 June 1987, see abstract ---	1-12
A	SCIENCE, vol. 249, 1990, pages 283-287, XP002020888 RÖTZSCHKE ET AL: "CHARACTERIZATION OF NATURALLY OCCURRING MINOR HISTOCOMPATIBILITY PEPTIDES INCLUDING H-4 AND H-Y" cited in the application ---	1-12
P,X	SCIENCE, vol. 269, 15 September 1995, pages 1588-1590, XP002020889 WANG ET AL: "HUMAN H-Y: A MALE-SPECIFIC HISTOCOMPATIBILITY ANTIGEN DERIVED FROM THE SMCY PROTEIN" see the whole document ---	1-12
P,X	NATURE, vol. 376, 24 August 1995, pages 695-698, XP002020890 SCOTT ET AL: "IDENTIFICATION OF A MOUSE MALE-SPECIFIC TRANSPLANTATION ANTIGEN, H-Y" see the whole document ---	1-6,12
P,X	SCIENCE, vol. 269, 15 September 1995, pages 1515-1516, XP002020891 PENNISI: "LONG-SOUGHT H-Y ANTIGEN FOUND" see the whole document -----	1-12

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/ 12351

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-11
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-11 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.